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METAL CATALYSTS IN BIOLOGIC OXIDATIONS

TISSUE INHIBITORS

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE PHYSICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

1933

By
AUSTIN BIRDINE WILDER

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METAL CATALYSTS IN BIOLOGIC OXIDATIONS

TISSUE INHIBITORS

By A. B. WILDER

The rate at which a tissue burns substrate and frees energy is, for it, a basic datum and may be said to measure its "speed of life" (Gerard, 1932). This rate, though subject to great increase during activity, is remarkably constant under ordinary conditions of rest. Yet little attention has been paid to the quantitative control of oxidations at rest, and less to the problem of an increase during function. If the skeleton reaction

$$\text{Substrate} + \text{oxygen} + \text{catalyst} \rightarrow \text{oxidized products}$$

be considered, it is easily seen that the reaction velocity, according to the mass action law, might vary with the concentration of reactants and products. In extreme cases it is true that these may play a critical rôle, but ordinarily they do not. Thus, when the oxygen concentration is greatly diminished, cell respiration begins to decrease; when substrate is withheld, especially for simple bacterial cells, the same is true; and when CO_2 or other acid is allowed to accumulate in excess, respiration is again slowed. Yet under conditions approaching normal, any or all of these factors may be widely varied without altering the respiratory rate of the resting cell. The critical factors must be, then, the amount

of active catalytic system and the conditions determining its accessibility to substrate, degree of activity, etc.

As oxidation catalysts, metal complexes, especially the iron porphyrins, are recognized as playing an outstanding part. Though attempts to correlate the metal content with the respiratory rate of a tissue have been suggestive, a strict parallel surely does not hold; and most of the iron present, for example, is at any one time catalytically inert (Warburg and Kubowitz, 1928). Further, sudden changes in respiratory rate could not depend on similar changes in metal content. One would expect in cells, therefore, secondary systems able to play upon the primary catalysts so as to maintain a constant activity or abruptly increase (and, possibly less abruptly, decrease) it. These systems might have to do mainly with structural organization (as, *e.g.*, adrenalin frees diastase or glycogenase from absorption in the salivary or liver cell (Lesser, 1921, 1927); and changes in colloidal "carriers" result from many conditions (Willstätter and Rohdewald, 1934)) or might act more directly upon the catalytic agent. In case of the latter possibility, cells would contain substances able to enhance or depress the oxidative catalyses induced by metals. Recent evidence suggests that urease, papain, and the glycolytic enzyme of muscle contain the sulfhydryl group and are reversibly inactivated by oxidizers, reactivated by reducers (Michaelis and Runnström (1934); see for other literature). Further, a coenzyme, often complex, is required for most glycolyses and oxidations (Andersson, 1934), and a common component of these systems, adenylpyrophosphate (Lohmann, 1931), is itself formed and destroyed in a complicated metabolic sequence (Parnas, Ostern, and Mann, 1934). These mechanisms all are capable of acting to regulate the metabolic flow of cells. More particularly, Michaelis and Solomon (1931) have obtained respiration accelerators from tissues, and it seemed a reasonable expectation that tissue inhibitors for metal catalyses should also be present. The presence of specific inhibitors is here reported. Of especial interest is a copper inhibitor in liver. Preliminary studies indicate that, in different samples, the concentration of this inhibitor runs parallel to the therapeutic value of the liver extract in pernicious anemia. A rôle of copper in hemoglobin formation has long been known (Elvehjem, 1935).

Inhibitors might act by forming less active complexes with the

active metals and could be tested for, therefore, by observing a diminution of metal catalysis in their presence. Since the quantity of active metal in tissues is itself small, the secondary substances might be present in minute amounts. The catalytic system described in Paper I adapts itself admirably, however, to the investigation of minute quantities of active substances. The small thiol and metal concentrations used in our test system are favorable from this view-point. Appropriate blanks have, of course, been performed to allow for the metal content in the tissue preparations themselves. Some simple inhibitors were also tested before the examination of tissues was undertaken. The phosphates, discussed in Paper I, belong in part in such a class.

Methods

Technique—The methods employed for the measurement of rates of oxidation are described in detail in Paper I. There, also, is described the method used for expressing those rates. In the present experiments no fundamental changes in the procedure were required, and the extracts or other substances were added to the test solution as it was prepared with no untoward difficulty. The pH of the solution was adjusted in the usual manner, or when colored extracts precluded the colorimetric method, a hydrogen electrode was used.

To avoid weighing exceedingly small amounts of material, stock solutions, from which aliquots were drawn, were used in many cases.

Preparation of Extracts—To obtain reproducible results, certain precautions must be taken in handling the tissue extracts. The tissue must be extracted while fresh. At first tumor material was collected during a week (50 hens were inoculated for each batch) and kept frozen. The extracts, finally obtained, were then cloudy and gave atypical results. Presumably the freezing and thawing released additional material. The final extracts should be kept free of moisture and oxygen, within reasonable limits, and will then yield reproducible results for at least 4 months. After 6 months the results become unreliable.

The method outlined was employed in the preparation of the muscle, liver, and tumor extracts. The absolute, but not relative, amounts used may of course be varied.

200 gm. of tissue are ground into 60 cc. of H_2O and stirred for

half an hour. 295 cc. of 95 per cent ethyl alcohol are added, the mixture centrifuged, the residue resuspended in 115 cc. of the alcohol, stirred a short time, and again centrifuged. The centrifugates are combined and the solvents removed under reduced pressure, during which the temperature is not allowed to exceed 35°. The residual material is dried *in vacuo* over phosphoric anhydride and powdered. It weighs about 6 gm.

For the preparation of saline-extracted samples, tumor tissue is chopped coarsely into 50 cc. of 0.9 per cent sodium chloride solution, stirred for 2 to 3 minutes, and centrifuged; the residue is washed with 25 cc. of H_2O and again centrifuged. The washings are discarded. The residue is now treated as above. The preliminary washing serves to remove extracellular débris.

Results

Simple Inhibitors—The catalytic action of copper is inhibited by methyl isocyanide (R—NC) about as powerfully as by the inorganic cyanides. 2×10^{-5} M KCN, for example, diminishes the action of 10^{-5} M Cu^{++} by 14 per cent; CH_3NC, by 8 per cent; and 10 times this concentration of the isocyanide gives over 98 per cent inhibition. The isomeric methyl cyanide (R—CN), on the other hand, is ineffective even in 0.01 M concentration. Tartaric acid is about one-fourth as powerful an inhibitor for copper as is the isocyanide—at molar ratios to copper of 10:1, the former causes an 11 per cent, the latter a 46 per cent decrease—a finding in harmony with the general relative stability of the two complexes formed. Cupferron, forming a slightly soluble copper complex (Baudisch, 1909), gives an 8 per cent inhibition (molar ratio to Cu 20:1). Glycine, in small concentration, has no effect, though it forms a complex inner salt with copper (Borsook and Thimann, 1932), and in large amounts, 0.3 M, gives an actual acceleration. Sodium dimethyl dithiocarbamate, containing non-oxidizable sulfur groups, forms a very stable copper complex (cherry-red) and inhibits thioglycolic acid oxidation as powerfully as does the isocyanide.

Since colloidal tissue preparations were to be examined, it was necessary to test the possible inhibitory action of colloids. Gelatin (3×10^{-4} M) depresses copper activity by 14 per cent, does not affect iron, and increases that of manganese (owing, possibly, to a

copper impurity in it). Egg albumin, on the other hand, strongly inhibits copper and manganese (iron was not tested), the former

TABLE I

Effect of Various Substances on Rate of Oxidation of Thioglycolic Acid in Presence of Metals (Titrimetric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7; oxygen supplied at 4 liters per hour. Rate expressed in terms of (moles of R—SH oxidized $\times 10^6$)/(time in minutes).

Inhibitor	Concentration	Catalyst	Rate	Change in rate
	M	*M* $\times 10^5$		*per cent*
		0	0.7	
		1 Cu	122	
		0.5 "	49	
		4.0 Fe	36	
		0.5 Mn	77	
Potassium cyanide	2×10^{-5}	1 Cu	105	−14
Methyl isocyanide	2×10^{-5}	1 "	112	−8
" "	1×10^{-4}	1 "	56	−54
" "	2×10^{-4}	1 "	3	−98
" "	1×10^{-2}	1 "	0	−100
" cyanide	1×10^{-2}	1 "	119	−2
Tartaric acid	5×10^{-5}	0	4	
" "	5×10^{-5}	0.5 Cu	48	−10
Cupferron	5×10^{-5}	0	7	
"	5×10^{-5}	0.5 Cu	57	+2
"	1×10^{-4}	0.5 "	52	−8
Glycine	6×10^{-5}	1 "	119	−2
"	3×10^{-1}	0.5 "	80	+63
Sodium dimethyl dithiocarbamate	2×10^{-5}	1 "	104	−15
Potassium iodide	4×10^{-2}	1 "	52	−57
Egg albumin	4×10^{-4}	1 "	38	−69
" "	4×10^{-4}	0.5 Mn	45	−42
Gelatin	3×10^{-4}	0	13	
"	3×10^{-4}	0.5 Cu	55	−14
"	3×10^{-4}	4 Fe	50	+3
"	3×10^{-4}	0.5 Mn	132	+55
		10 $K_4Fe(CN)_6$	4	

about as powerfully as the isocyanide. It seems to follow that colloids or proteins *per se* do not inhibit, though certain members of

the group may do so. This point is reinforced by the findings with particular tissue extracts which exhibit specific and individual inhibitory effects.

Table I summarizes the effects of these substances on the heavy metal catalyses of thioglycolic acid oxidation, under varied conditions. Each datum represents several experiments, none of which varied by more than ±5 per cent from the mean. For purposes of comparison, the rates in the absence of inhibitors are placed at the head of the table. It will be observed that in the absence of heavy metal ions, the rate of oxidation of thioglycolic acid is negligible.

Muscle—Beef or chicken muscle, ground in water and extracted with alcohol, yields finally a small amount of powder which is strongly inhibitory to iron. The same material increases the activity of copper or manganese, possibly because of the presence in it of these metals and their consequent mutual acceleration (*cf.* Paper I). Copper and iron added together sum their separate effects in the presence of the extract as well as in simple buffers; and since added iron exhibits only 40 to 50 per cent of its normal activity, the presence of a specific inhibitor for this ion is indicated (Table II). The inhibitor could hardly be a phosphate in view of the activity of manganese, for in all cases manganese is more sensitive than iron to phosphate inhibition.

Note that the extract of chicken muscle inhibits the iron catalysis more than does a 4 times more concentrated extract of beef muscle. The acceleration by extract alone, conversely, is distinctly less for chicken muscle, even at an equivalent concentration.

Sarcoma—Rous sarcoma, obtained by injection into the pectoral muscles of Plymouth Rock hens of the Rous powder (generously supplied by Dr. Murphy of the Rockefeller Institute), behaved much like the muscle in which it developed. Although the tumor tissue was meticulously separated from normal muscle, necrotic fragments, clotted blood, mucoid material, and the like, the final product must be regarded as composed of only relatively pure sarcoma cells. When this material is extracted in the same manner as muscle, the iron inhibitor (as well as the copper or manganese accelerators) is again found, but the inhibitory effect is less intense than with muscle (Table III). The inhibition observed is surely more than could be exerted by any contaminating

TABLE II

Inhibition by Muscle Extracts of Iron Catalysis of Thioglycolic Acid Oxidation

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. Muscle extract present in concentration of 11 gm. per liter of thioglycolic acid solution. (Refer to Table I for separate effects of metal ions.)

Muscle extract added	Catalyst	Rate: Moles R—SH oxidized × 10^5 / Time in min.	Change in rate
	Titrimetric measurements		
	M × 10^5		*per cent*
Beef	0	27	
"	0.5 Cu^{++}	102	+54
"	4 Fe^{++}	45	−50
"	0.5 Mn^{++}	123	+25
"	0.5 Cu^{++} + 4Fe^{++}	122*	
	Manometric measurements		
		O_2 absorbed per 100 min.	
		c.mm.	
	10 Fe^{++}	90	
Beef	0	48	
"	10 "	91	−52

Manometric measurements. Experimental constants, as above, except concentration of extract is smaller, 2.5 gm. per liter of thioglycolic acid solution.

	0.5 Cu^{++}	45	
	10.0 Fe^{++}	90	
	0.5 Mn^{++}	55	
Chicken		8	
"	0.5 Cu^{++}	64	+45
"	10 0 Fe^{++}	43	−61
"	0.5 Mn^{++}	95	+58

* Copper ions do not affect iron catalysis. Thus:

Rate for extract and copper ions	102
" " iron in presence of extract (50% of 36)	18
Total	120

Compare with the observed value of 122.

muscle present, and we conclude that the iron inhibitor is present in reduced amount in the cancer cells. Consistent results were obtained with six separate lots of sarcoma. It is suggestive that

in cancer, which is characterized by a loss of controlled or restrained growth, by increased glycolysis, and the like, there is a decrease in an inhibitory agent for iron, as compared to its tissue of origin. This is the more interesting since iron catalysts are

TABLE III

Effect of Rous Sarcoma Extracts on the Rate of Metal-Catalyzed Thiol Oxidation (Manometric Measurements)*

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. Concentration of extracts 2.5 gm. per liter of solution.

Substance added	Catalyst	O_2 absorbed per 100 min.	Change in rate
	M × 10^5	*c.mm.*	*per cent*
None	0.5 Cu^{++}	45	
"	10.0 Fe^{++}	90	
"	0.5 Mn^{++}	55	
Rous sarcoma, Extract A		17	
" " " "	0.5 Cu^{++}	72	+22
" " " "	10.0 Fe^{++}	92	−17
" " " "	0.5 Mn^{++}	138	+120
" " " 1-B		3	
" " " 1-B	0.5 Cu^{++}	96	+107
" " " 1-B	10.0 Fe^{++}	65	−31
" " " 1-B	0.5 Mn^{++}	129	+129
" " " 2-B		11	
" " " 2-B	0.5 Cu^{++}	89	+73
" " " 2-B	10.0 Fe^{++}	34	−71
" " " 2-B	0.5 Mn^{++}	22	−80

* Extract A is the standard preparation; Extract 1-B, the one after saline washing of the tumor. Extract 1-B probably contains less metal, since it induces a lower rate of oxidation of thioglycolic acid. The sarcoma Extract 2-B was prepared as for Extract 1-B from tissue kept frozen over a week. In this case manganese catalysis is inhibited, whereas extracts prepared from fresh tumor always accelerate. The inhibition may well be due to inorganic phosphates produced by the hydrolysis of organic phosphates. In harmony with this possibility, iron catalysis is inhibited also, but to a lesser degree than manganese (see Paper I).

apparently involved in glycolysis (non-hemin Fe^{++}) (Zuckerkandl, Fleischmann, and Drucker, 1934) as well as in several oxidation systems.

The catalytic action of the tumor extract without added metals is high, more than that of muscle, which suggests a large metal

content. This is probably due to necrotic tissue and blood not fully removed from the sarcoma. As evidence for this, if the fresh tissue is coarsely chopped and washed once with isotonic saline solution before the usual extraction, its intrinsic catalytic action is largely lost, while its other effects are unaltered. Since this washing should remove soluble material not held by the semi-permeable membranes of living cells, the bulk of the catalytic effect of the extract is presumably derived from extracellular material, the specific inhibitor from the living cells. Control experiments on blood (see Table VI) further support the view that

TABLE IV

Effect of Liver Extracts on the Metal Catalysis of Thiol Oxidation (Manometric Measurements)

Temperature, 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8 7; 2.5 gm. of liver extract per liter of solution.

Substance added	Catalyst	O_2 absorbed per 100 min.	Change in rate
	$M \times 10^5$	*c.mm.*	*per cent*
	0.5 Cu^{++}	45	
	10.0 Fe^{++}	80	
	0.5 Mn^{++}	55	
Chicken liver, Extract A		15	
" " " "	0.5 Cu^{++}	42	−40
" " " "	10.0 Fe^{++}	96	+1
" " " "	0.5 Mn^{++}	148	+142

specific cellular substances are involved in the inhibitory action of tissues.

Liver—Chicken liver was extracted by means of the identical procedure applied to the other materials, yet the liver extract affected the metal catalyses in an entirely different manner. This fact greatly enhances the probability that specific substances, rather than some overlooked feature of the general conditions, are responsible for the findings. Liver extract is entirely neutral to iron and inhibits copper, while manganese, as in the other cases, is accelerated (Table IV).

Liver extracts prepared in a variety of ways exhibit a wide variation in their effectiveness as copper inhibitors (I am indebted to Eli Lilly and Company for supplying us with a

TABLE V

Effect of Liver Extracts on Metal Catalysis of Thiol Oxidation (Titrimetric Measurements)

Temperature 20°; 0.014 M thioglycolic acid; carbonate buffer, pH 8.7. The extract was present in concentration of 11 gm. per liter of solution.

Extract No.	O_2 absorbed per 100 min. (no metal added)	Catalyst	O_2 absorbed per 100 min. (with catalyst)	Change in rate	Clinical results
	c.mm.	*M* × 10^5	*c.mm.*	*per cent*	
		0.5 Cu^{++}	49		
		1.0 "	122		
		2.0 "	270		
		4.0 Fe^{++}	35		
		0.5 Mn^{++}	77		
Liver					
841015	11	0.5 Cu^{++}	45	−31	+
841015 (digested with concentrated HCl)	134	0.5 "	196	+27	
841015	11	4.0 Fe^{++}	31	−43	
841015	11	0.5 Mn^{++}	97	+12	
E-191	10	0.5 Cu^{++}	41	−37	
E-197	13	0.5 "	17	−92	
E-197	13	1.0 "	22	−93	
E-197	13	2.0 "	49	−87	
E-330-B	46	0.5 "	95	0	
E-352	15	0.5 "	25	−80	+
E-352	15	0.5 Mn^{++}	148	+73	
E-353	20	0.5 Cu^{++}	87	+37	
E-374-A	28	0.5 "	97	+41	−
E-374-B	34	0.5 "	41	−86	
E-375	49	0.5 "	84	−29	
E-377-A	17	0.5 "	46	−41	
E-377-B	81	0.5 "	136	+12	−
E-379-A	31	0.5 "	87	+14	−
Kidney					
E-196	78	0.5 "	186	+120	
Spleen					
E-198	57	0.5 "	118	+24	

number of such extracts).[1] These extracts were prepared in connection with the therapy of pernicious anemia and several had been assayed for therapeutic action in the Lilly laboratories by means of their usual clinical tests. It was found, on comparing our assay of the inhibitory action of these several extracts on copper catalysis with such of their results on therapeutic efficacy as were available, that the two properties ran parallel. Liver preparations that failed to inhibit copper also failed to improve pernicious anemia patients; those that did inhibit copper improved patients. A few experiments with spleen and kidney extracts prepared along similar lines failed to demonstrate anti-catalytic activity. Some results are collected in Table V.

TABLE VI

Effect of Blood Extract on Metal Catalysis of Thiol Oxidation (Manometric Measurements)

The experimental constants were the same as for Table III, except that 0.5 gm. of extract per liter of solution was used.

Substance added	Catalyst	O_2 absorbed per 100 min.	Change in rate
	$M \times 10^5$	*c.mm.*	*per cent*
	0.5 Cu^{++}	45	
	10.0 Fe^{++}	90	
	0.5 Mn^{++}	55	
Blood extract		9	
" "	0.5 Cu^{++}	68	+29
" "	10.0 Fe^{++}	117	+17
" "	0.5 Mn^{++}	192	+232

This parallelism, of course, suggests the availability of the catalytic method for assaying the clinical value of liver extracts, and the experiments so far conducted warrant further study and a more complete separate report. Further, these results again emphasize the specific identity of catalytic inhibitors (and, less surely, accelerators) present in the various body cells.

Blood—Fresh blood was extracted by the usual method, 1500 gm. of sheep blood yielding 8 gm. of extract. Its effect on heavy metal catalyses is illustrated in Table VI.

[1] Some of these extracts showed also a strong inhibitory effect upon iron. This effect could be directly traced to an extraction procedure which allowed the introduction of specific iron inhibitors.

The effects of the blood extract on copper, iron, and manganese ion catalyses are accelerations of 29, 17, and 232 per cent respectively. It is possible from these data to estimate the influence of blood contained in the sarcoma tissues investigated. Assuming that 10 per cent of the fresh weight is blood (surely a generous figure), 20 gm. of blood would be extracted in the usual 200 gm. sample. This blood would yield 0.1 gm. of extract to 5 gm. for the whole sample, or 2 per cent. 5 mg. of the whole, used in a test, would contain 0.1 mg. of blood extract, which, by extrapolation from the above results with 1 mg., would accelerate copper 4 per cent, iron 2 per cent, and manganese about 20 per cent. Except for the last, such effects are negligible and, since all are in a positive direction, they could not account for the specific inhibitory effect of sarcoma extracts.

SUMMARY

1. The ability of tissue extracts as well as of a group of specific substances to enhance or decrease the rate of metal-catalyzed oxidation of thioglycolic acid was studied by the methods described in Paper I.

2. A number of chemicals able to form complexes with copper ion inhibit its catalytic action to an extent roughly paralleling the stability of the complex. Colloidal materials, as such, are inactive.

3. Beef or chicken muscle yields extracts highly inhibitory to iron, somewhat acceleratory to copper and manganese.

4. Chicken sarcoma (Rous) extracts act like muscle, but are less powerful.

5. Chicken liver extracts are neutral to iron, accelerate manganese, and inhibit copper. In a few cases where direct comparison was made, the intensity of copper inhibition produced by various extracts of liver paralleled their therapeutic value in the treatment of pernicious anemia.

6. Control tests with blood extracts exclude blood as a factor in the above results.

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